(12) INTERNATIONAL [redacted]ICATION PUBLISHED UNDER THE PATENT [redacted]PERATION TREATY (PCT)

(19) World Intellectual Pr perty Organization
International Bureau

(43) International Publication Date
1 February 2001 (01.02.2001)

PCT

(10) International Publication Number
WO 01/07570 A1

(51) International Patent Classification[7]: C12N 5/04, 15/29, 15/82, C07H 21/04, A01H 5/00, C12P 19/00

(21) International Application Number: PCT/US00/19981

(22) International Filing Date: 21 July 2000 (21.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/359,161 21 July 1999 (21.07.1999) US

(71) Applicant: **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, 5th floor, Oakland, CA 94607-5200 (US).

(72) Inventors: **BRADFORD, Kent, J.**; 1003 Miller Drive, Davis, CA 95616 (US). **PEETAMBAR, Dahal**; 4500 Alhambra Drive # 152, Davis, CA 95616 (US). **YANG, Hong**; 945 Lucena Court, Davis, CA 95616 (US). **COOLEY, Michael**; 1536 Shannon Court, Benecia, CA 94510 (US). **DOWNIE, Bruce**; 215 Arcadia Park, Lexington, KY 40503 (US). **GEE, Oliver, Henry**; 744 Sycamore Lane, Davis, CA 95616 (US).

(74) Agents: **BASTIAN, Kevin, L.** et al.; Townsend and Townsend and Crew LLP, Two Embarcadero Center, 8th floor, San Francisco, CA 94111-3834 (US).

(81) Designated States *(national)*: CA, JP.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/07570 A1

(54) Title: REGULATION OF SOURCE-SINK RELATIONSHIPS AND RESPONSES TO STRESS CONDITIONS IN PLANTS

(57) Abstract: The invention provides the identification and characterization of plant *SNF4* and *SNF1* genes. Examples of the genes were cloned from tomato. They are nucleic acids and proteins belonging to the SNF1-related protein kinase family, and are involved in plant's response to stress conditions such as nutritional and environmental stresses. The invention also provides transgenic plants containing the genes, and methods of modulating stress responses in transgenic plants comprising these genes.

REGULATION OF SOURCE-SINK RELATIONSHIPS AND RESPONSES TO STRESS CONDITIONS IN PLANTS

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This invention was made with Government support under Grant Nos. IBN-9407264 and UBN-9722978 awarded by the National Science Foundation. The Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Cells must sense their nutritional or environmental conditions and modify their metabolic activity appropriately. Yeast SNF1 (sucrose non-fermenting) protein kinase and mammalian AMP-activated protein kinase (AMPK) are central components of kinase cascades that act as metabolic sensors of glucose availability and AMP:ATP levels respectively. Protein sequence and functional homology exists between the yeast and mammalian kinase subunits (SNF1/AMPK-α), activation subunits (SNF4/AMPK-γ) and the docking subunits (SIP/AMPK-β) that constitute the functional kinase complexes (Hardie, D., *et al.*, *Annu. Rev. Biochem.* 67:821-55 (1998)).

In yeast, the association of the SNF4 activating subunit with a regulatory region of the SNF1 protein is sensitive to glucose. When glucose concentration is low, the SNF4 protein associates with the regulatory domain of SNF1, and the activity of the catalytic kinase domain is increased, resulting in the derepression of genes required for the metabolism of alternative energy sources. When glucose concentration is high, the SNF1 kinase domain associates with its regulatory domain and kinase activity is inhibited. In mammals, the activation of AMPK, in response to increases in the AMP:ATP ratio, results in the switching on of ATP-producing pathways and the switching off of ATP-consuming pathways. For example, AMPK activation results in the phosphorylation and inactivation of acetyl coenzyme A carboxylase and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoA reductase); but unlike in yeast, the specific functions of the γ- and β-subunits are less well defined.

In plants, there is also evidence that carbohydrates control gene expression, growth, metabolism and differentiation. Jang and Sheen, *Trends in Plant Sciences*, 2:208-214 (1997); Koch, *Annu. Rev. Plant. Physiol. Mol.*, 2:509-540 (1996)). An extensive family of SNF1 homologs and related kinases have been characterized and have been grouped into several subfamilies of SNF1-related kinases (SnRKs) (Halford, *et al.*, *Plant Mol. Biol.* 37:735-748 (1996)). In addition to exhibiting kinase activity on substrates common to the mammalian and yeast kinases and complementing yeast SNF1 mutants (Alderson, *et al. Proc. Natl. Acad. Sci. U.S.A.* 88:8602-05 (1991); Muranaka, *et al. Mol. Cell Biol.*, 14:2958-65 (1994)), antisense suppression experiments suggest that plant SNF1 homologs may also be involved in regulation of carbon metabolism *in planta*. Purcell, *et al., Plant J.* 14:195 (1998). However, NPK5, a SNF1 homolog from tobacco was unable to complement an SNF4-deletion yeast mutant strain (*Δ-SNF4*), suggesting that NPK5 may require an SNF4-like component for physiological activity *in vivo* (Muranaka *et al.*, *Mol Cell Biol.*, 14:2958-65 (1994)).

No functional homolog of the SNF4 activating subunit has yet been demonstrated from plants, although a gene sequence (Pv42) isolated from developing bean seeds was reported to have predicted amino acid sequence similarity to SNF4. (Accession No: U40713.) Thus, there exist needs to identify and express plant homologs to yeast SNF4 proteins in order to understand how plants cope with metabolic and stress conditions in the environment and to modulate these responses to engineer plants resistant to various environmental stresses. In addition, production of genetically engineered plants with improved carbon metabolism and source-sink relationships could be used to improve yields or qualities of harvested plant products. The present invention addresses these and other needs.

SUMMARY OF THE INVENTION

The present invention provides SNF4 homologs from plants. In particular, the present invention provides nucleic acid molecules which encode plant SNF4 polypeptides. The polypeptides of the invention comprise an amino acid sequence that has greater than about 70% identity to SEQ ID NO: 3.

Also provided is the promoter sequence from SEQ ID NO: 2. Promoters of the invention can be operably linked to heterologous nucleic acid sequences and used to drive expression of the heterologous sequences in desired plant tissues.

The present invention further provides SNF1 polypeptides. The SNF1 polypeptides of the invention comprise an amino acid sequence that has greater than about 95% identity to the amino acid sequence of the polypeptide encoded by SEQ ID NO: 4. An exemplary SNF1 nucleic acid molecule from tomato is shown in SEQ ID NO: 4 (LeSNF1). Preferably, the nucleic acid molecule can specifically hybridize to SEQ ID NO: 4 or its complement.

The present invention further provides recombinant expression vectors comprising the nucleic acid sequences of the invention. Preferably, the vectors comprise a plant promoter operably linked to the nucleic acid sequence. The promoter can be either a constitutive promoter, or an inducible promoter.

The present invention also provides for transgenic plants comprising a recombinant expression cassette of the invention. The recombinant expression cassettes are useful in methods of modulating source-sink relationships in plants and thereby enhancing yield or quality of harvested plant products, such as fruit. For example, the nucleic acids of the invention can be used to enhance sink activity and starch or lipid accumulation in seeds. Alternatively, the can be used to enhance sugar accumulation in fruit. The expression cassettes of the invention can also be used to enhance responsiveness to stress conditions in plants.

DEFINITIONS

The term "stress conditions" as used herein generally refers to nutritional and environmental stress that plants encounter in their life cycle. Examples of stress conditions are any nutritional or environmental changes that lead to changes in plant internal metabolic pathways and alterations in the plant's carbon reserves. Examples of environmental stresses include extreme temperature (*e.g.* excess heat or cold), high salt, flooding, anoxia, drought, toxic chemicals (*e.g.* herbicides, heavy metals) and the like.

The term "plant SNF4 polypeptide" refers to plant homologs of yeast SNF4. Without wishing to be bound by theory it is believed that the polypeptides of the invention are activating subunits in kinase cascades that act as metabolic sensors of carbohydrate availability and ATP levels in plant cells The proteins of the invention are a component in SNF1 related protein kinases which are composed of kinase subunits (SNF1), activation subunits (SNF4), and docking subunits (SIP). The term "LeSNF4" refers to plant SNF4 polypeptides derived from tomato (*Lycopersicon esculentum*).

Plant SNF4 polypeptides of the invention are typically from about 20 amino acids to about 400 amino acids in length, usually from about 100 to about 375, and

often from about 200 to about 300 amino acids. A full length plant SNF4 polypeptide of the invention is typically about 375 amino acids.

The term "plant SNF1 polypeptide" refers to a plant homolog of the SNF1 subunit of the SNF1-related protein kinase. An example of a SNF1 nucleic acid is the LeSNF1 nucleic acid sequence as shown in SEQ ID NO.: 4. An example of a SNF1 amino acid sequence is the LeSNF1 amino acid sequence as shown in SEQ ID NO.: 5.

The phrase "nucleic acid sequence" refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes chromosomal DNA, self-replicating plasmids, infectious polymers of DNA or RNA and DNA or RNA that performs a primarily structural role.

The term "promoter" refers to regions or sequence located upstream and/or downstream from the start of transcription and which are involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells. Such a promoter can be derived from plant genes or from other organisms, such as viruses capable of infecting plant cells.

The term "plant" includes whole plants, shoot vegetative organs/structures (*e.g.* leaves, stems and tubers), roots, flowers and floral organs/structures (*e.g.* bracts, sepals, petals, stamens, carpels, anthers and ovules), seed (including embryo, endosperm, and seed coat) and fruit (the mature ovary), plant tissue (*e.g.* vascular tissue, ground tissue, and the like) and cells (*e.g.* guard cells, egg cells, trichomes and the like), and progeny of same. The class of plants that can be used in the method of the invention is generally as broad as the class of higher and lower plants amenable to transformation techniques, including angiosperms (monocotyledonous and dicotyledonous plants), gymnosperms, ferns, and multicellular algae. It includes plants of a variety of ploidy levels, including aneuploid, polyploid, diploid, haploid and hemizygous.

A polynucleotide sequence is "heterologous to" an organism or a second polynucleotide sequence if it originates from a foreign species, or, if from the same species, is modified from its original form. For example, a promoter operably linked to a heterologous coding sequence refers to a coding sequence from a species different from that from which the promoter was derived, or, if from the same species, a coding sequence which is not naturally associated with the promoter (*e.g.* a genetically engineered coding sequence or an allele from a different ecotype or variety).

A polynucleotide "exogenous to" an individual plant is a polynucleotide which is introduced into the plant by any means other than by a sexual cross. Examples of means by which this can be accomplished are described below, and include *Agrobacterium*-mediated transformation, biolistic methods, electroporation, and the like. Such a plant containing the exogenous nucleic acid is referred to here as a T_1 (*e.g.* in *Arabidopsis* by vacuum infiltration) or R_0 (for plants regenerated from transformed cells *in vitro*) generation transgenic plant. Transgenic plants that arise from sexual cross or by selfing are descendants of such a plant.

"Recombinant" refers to a human manipulated polynucleotide or a copy or complement of a human manipulated polynucleotide. For instance, a recombinant expression cassette comprising a promoter operably linked to a second polynucleotide may include a promoter that is heterologous to the second polynucleotide as the result of human manipulation (*e.g.*, by methods described in Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989) or Current Protocols in Molecular Biology Volumes 1-3, John Wiley & Sons, Inc. (1994-1998)) of an isolated nucleic acid comprising the expression cassette. In another example, a recombinant expression cassette may comprise polynucleotides combined in such a way that the polynucleotides are extremely unlikely to be found in nature. For instance, human manipulated restriction sites or plasmid vector sequences may flank or separate the promoter from the second polynucleotide. One of skill will recognize that polynucleotides can be manipulated in many ways and are not limited to the examples above.

Two nucleic acid sequences or polypeptides are said to be "identical" if the sequence of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described below. The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence over a comparison window, as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. When percentage of sequence identity is used in reference to proteins or peptides, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions, where amino acids residues are substituted for other amino acid residues with similar chemical properties

(e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated according to, e.g., the algorithm of Meyers & Miller, *Computer Applic. Biol. Sci.* 4:11-17 (1988) e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA).

The phrase "substantially identical," in the context of two nucleic acids or polypeptides, refers to sequences or subsequences that have at least 60%, preferably 70%, more preferably 80%, most preferably 90-95% nucleotide or amino acid residue identity when aligned for maximum correspondence over a comparison window as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition also refers to the complement of a test sequence, which has substantial sequence or subsequence complementarity when the test sequence has substantial identity to a reference sequence.

One of skill in the art will recognize that two polypeptides can also be "substantially identical" if the two polypeptides are immunologically similar. Thus, overall protein structure may be similar while the primary structure of the two polypeptides display significant variation. Therefore a method to measure whether two polypeptides are substantially identical involves measuring the binding of monoclonal or polyclonal antibodies to each polypeptide. Two polypeptides are substantially identical if the antibodies specific for a first polypeptide bind to a second polypeptide with an affinity of at least one third of the affinity for the first polypeptide.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (*see generally, Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1995 Supplement) (Ausubel)).

Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-410 and Altschuel *et al.* (1977) *Nucleic Acids Res.* 25: 3389-3402, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al, supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a

comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)).

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

A further indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as described below.

"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill

will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

As to amino acid sequences, one of skill will recognize that individual substitutions, in a nucleic acid, peptide, polypeptide, or protein sequence which alters a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art.

The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

(*see, e.g.*, Creighton, *Proteins* (1984)).

An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below.

The phrase "selectively (or specifically) hybridizes to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (e.g., total cellular or library DNA or RNA).

The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of

nucleic acid, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, highly stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. Lower stringency conditions are generally selected to be about 15-30 °C below the T_m. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 time background hybridization.

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions.

In the present invention, genomic DNA or cDNA comprising plant *SNF4* or *SNF1* nucleic acids of the invention can be identified in standard Southern blots under stringent conditions using the nucleic acid sequences disclosed here. For the purposes of this disclosure, suitable stringent conditions for such hybridizations are those which include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and at least one wash in 0.2X SSC at a temperature of at least about 50°C, usually about 55°C to about 60°C, for 20 minutes, or equivalent conditions. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides that they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

A further indication that two polynucleotides are substantially identical is if the reference sequence, amplified by a pair of oligonucleotide primers, can then be used as a probe under stringent hybridization conditions to isolate the test sequence from a cDNA or genomic library, or to identify the test sequence in, *e.g.*, an RNA gel or DNA gel blot hybridization analysis.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the predicted amino acid sequences of LeSN4, Pv42, AMPK-γ and SNF4. Identical amino acids and conservative substitutions are shaded. CBS domains are underlined. Alignment was by the J. Hein method using a structural weight table (Megalign software, DNAStar, Inc., Madison WI).

Figures 2A and 2B are schematic representations of the yeast SNF1/SNF4 complex in yeast and homologs in plants and mammals. Figure 2A -In yeast, SNF1 and SNF4 interact to form a complex with one of a family of proteins (SIP1/SIP2/GAL83). Each of these latter proteins can independently interact with SNF4 at an association domain (ASC) and with SNF1 at a kinase interacting sequence (KIS). When glucose is high, interaction between the KD and RD of SNF1 autoinhibits its kinase activity. When glucose is low, SNF4 interacts with the RD of SNF1, activating the KD region. There is also evidence that SNF1 is phosphorylated (indicated by the P group) under derepressing conditions (low glucose) by a SNF1 activating factor homologous to a kinase known to activate AMPK in mammals. Figure 2B -The yeast SNF1 protein is composed of a kinase domain (KD) and a regulatory domain (RD). The regulatory domain can interact with the kinase domain of the same protein and the SNF4 protein. The NPK5 protein is a plant

homolog of SNF1 from tobacco. StubbGAK83 is a plant homolog from potato of the yeast SIP/GAL proteins (Lakatos *et al. Plant J.* 17:569-574 (1999)). Experimental evidence indicates that all the interaction shown with solid arrows occur *in vivo*, indicating conservation of these functions in plants and yeast. The α subunit of the mammalian AMP activated protein kinase (AMPK) is a homolog of SNF1, the γ-subunit is a homolog of SNF4and the β subunit is a homolog of SIP/GAL. This figure is updated from Jiang and Carlson *Genes Dev* 10:3105-3115 (1996).

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Introduction

The AMP-activated/SNF1 protein kinase family is conserved across mammals, plants and yeast and plays a critical role in cellular metabolic responses to nutritional or environmental stress. Members of this family are protein kinase components of kinase cascades that act as metabolic sensors of glucose availability and AMP: ATP levels (*see*, Figure 2). Structurally, SNF-1 related protein kinases are composed of kinase subunits (SNF1), activation subunits (SNF4), and docking subunits (SIP/GAL83), all three constituting the functional protein kinase complex. SNF4 activating subunits (such as those claimed here) associate with a regulatory region of the SNF1 protein, and regulate the activity of the protein kinase is response to sugar level. Since SNF1 protein kinases play a critical role in response to nutritional and environmental stress, the polynucleotides of the invention can be used to modulate source-sink relationships and responses to stress conditions in plants.

Increasing polypeptide activity or gene expression

Any of a number of means well known in the art can be used to increase activity of SNF1 or SNF4 polypeptides or polynucleotides of the invention in plants. Enhanced expression is useful, to alter expression of sugar related genes or to enhance resistance to stress. Any organ can be targeted, such as shoot vegetative organs/structures (*e.g.* leaves, stems and tubers), roots, flowers and floral organs/structures (*e.g.* bracts, sepals, petals, stamens, carpels, anthers and ovules), seed (including embryo, endosperm, and seed coat) and fruit. Alternatively, one or several genes of the invention can be expressed constitutively (*e.g.*, using the CaMV 35S promoter).

Usually isolated sequences prepared as described herein are used to prepare recombinant expression cassettes in recombinant vectors. The vectors are

introduced into plant cells using methods well known to those of skill in the art. Preparation of suitable constructs and means for introducing them into plants are described below.

One of skill will recognize that the polypeptides encoded by the nucleic acids of the invention, like other proteins, have different domains that perform different functions. Thus, gene sequences of the invention need not be full length, so long as the desired functional domain of the protein is expressed.

Modified protein chains can also be readily designed utilizing various recombinant DNA techniques well known to those skilled in the art and described in detail below. For example, the chains can vary from the naturally occurring sequence at the primary structure level by amino acid substitutions, additions, deletions, and the like. These modifications can be used in a number of combinations to produce the final modified protein chain.

Alternatively, endogenous *SNF4* or *SNF1* genes can be modified to enhance expression of these genes. Methods for introducing genetic mutations into plant genes and selecting plants with desired traits are well known. For instance, seeds or other plant material can be treated with a mutagenic chemical substance, according to standard techniques. Such chemical substances include, but are not limited to, the following: diethyl sulfate, ethylene imine, ethyl methanesulfonate and N-nitroso-N-ethylurea. Alternatively, ionizing radiation from sources such as, X-rays, fast neutrons or gamma rays can be used.

Alternatively, homologous recombination can be used to induce targeted gene modifications by specifically targeting gene of the invention *in vivo* (*see, generally*, Grewal and Klar, *Genetics* 146: 1221-1238 (1997) and Xu *et al.*, *Genes Dev.* 10: 2411-2422 (1996)). Homologous recombination has been demonstrated in plants (Puchta *et al.*, *Experientia* 50: 277-284 (1994), Swoboda *et al.*, *EMBO J.* 13: 484-489 (1994); Offringa *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 7346-7350 (1993); and Kempin *et al. Nature* 389:802-803 (1997)).

Other means for increasing activity of polynucleotides and polypeptides of the invention can also be used. For example, one method to increase expression of genes of the invention is to use "activation mutagenesis" (*see, e.g.* Hiyashi *et al. Science* 258:1350-1353 (1992)). In this method an endogenous gene of the invention can be modified to be expressed constitutively, ectopically, or excessively by

insertion of T-DNA sequences that contain strong/constitutive promoters upstream of the endogenous gene.

Inhibition of activity or expression of polynucleotides or polypeptides of the invention

Activity of endogenous *SNF1* or *SNF4* gene an also be inhibited using well known techniques. Inhibition of expression of these genes can be used, for instance, to modulate the activity of enzymes associated with sugar metabolism. For example, inhibition of these genes can be used to inhibit sucrose synthase and inducibility of this enzyme (*see, e.g.* Purcell *et al. Plant Journal* 14:195-202 (1998)). In seeds, inhibition of SN4 expression can be used to break dormancy and stimulate germination. Selective or tissue specific inhibition can be used to alter carbon metabolic pathways, for

The nucleic acid sequences disclosed here can be used to design nucleic acids useful in a number of methods to inhibit expression of genes of the invention in plants. For instance, antisense technology can be conveniently used. To accomplish this, a nucleic acid segment from the desired gene is cloned and operably linked to a promoter such that the antisense strand of RNA will be transcribed. The construct is then transformed into plants and the antisense strand of RNA is produced. In plant cells, it has been suggested that antisense suppression can act at all levels of gene regulation including suppression of RNA translation (*see*, Bourque, *Plant Sci. (Limerick)* 105: 125-149 (1995); Pantopoulos, In Progress in Nucleic Acid Research and Molecular Biology, Vol. 48. Cohn, W. E. and K. Moldave (Ed.). Academic Press, Inc.: San Diego, California, USA; London, England, UK. p. 181-238; Heiser *et al.*, *Plant Sci. (Shannon)* 127: 61-69 (1997)) and by preventing the accumulation of mRNA which encodes the protein of interest, (*see,* Baulcombe, *Plant Mol. Bio.* 32:79-88 (1996); Prins and Goldbach, *Arch. Virol.* 141: 2259-2276 (1996); Metzlaff *et al. Cell* 88: 845-854 (1997), Sheehy et al., *Proc. Nat. Acad. Sci. USA*, 85:8805-8809 (1988), and Hiatt et al., U.S. Patent No. 4,801,340).

The nucleic acid segment to be introduced generally will be substantially identical to at least a portion of the endogenous gene or genes (e.g., *LeSNF4*, or *LeSNF1*, etc.) to be repressed. The sequence, however, need not be perfectly identical to inhibit expression. The vectors of the present invention can be designed such that the inhibitory effect applies to other genes within a family of genes exhibiting identity or substantial identity to the target gene.

For antisense suppression, the introduced sequence also need not be full length relative to either the primary transcription product or fully processed mRNA.

Generally, higher identity can be used to compensate for the use of a shorter sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and identity of non-coding segments may be equally effective. Normally, a sequence of between about 30 or 40 nucleotides to about the full length of a nucleotide should be used, though a sequence of at least about 100 nucleotides is preferred, a sequence of at least about 200 nucleotides is more preferred, and a sequence of about 500 to about 3500 nucleotides is especially preferred.

A number of gene regions can be targeted to suppress expression of genes of the invention. The targets can include, for instance, the coding regions, introns, sequences from exon/intron junctions, 5' or 3' untranslated regions, and the like.

Another well-known method of suppression is sense co-suppression. Introduction of nucleic acid configured in the sense orientation has been recently shown to be an effective means by which to block the transcription of target genes. For an example of the use of this method to modulate expression of endogenous genes (*see*, Assaad *et al.*, *Plant Mol. Bio.* 22: 1067-1085 (1993); Flavell, *Proc. Natl. Acad. Sci. USA* 91: 3490-3496 (1994); Stam *et al.*, *Annals Bot.* 79: 3-12 (1997); Napoli et al., *The Plant Cell* 2:279-289 (1990); and U.S. Patents Nos. 5,034,323, 5,231,020, and 5,283,184).

The suppressive effect may occur where the introduced sequence contains no coding sequence *per se*, but only intron or untranslated sequences homologous to sequences present in the primary transcript of the endogenous sequence. The introduced sequence generally will be substantially identical to the endogenous sequence intended to be repressed. This minimal identity will typically be greater than about 65%, but a higher identity might exert a more effective repression of expression of the endogenous sequences. Substantially greater identity of more than about 80% is preferred, though about 95% to absolute identity is most preferred. As with antisense regulation, the effect should apply to any other proteins within a similar family of genes exhibiting identity or substantial identity.

For co-suppression, the introduced sequence, needing less than absolute identity, also need not be full length, relative to either the primary transcription product or fully processed mRNA. This may be preferred to avoid concurrent production of some plants that over-express the introduced sequence. A higher identity in a sequence shorter than full-length compensates for a longer, less identical sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and identity of non-coding segments will be equally effective. Normally, a sequence of the size ranges noted

above for antisense regulation is used. In addition, the same gene regions noted for antisense regulation can be targeted using co-suppression technologies.

Other means of inhibiting expression are known. These methods include formation of triple-helix DNA (*see, e.g.*, Havre and Glazer *J. Virology* 67:7324-7331 (1993); Scanlon *et al. FASEB J.* 9:1288-1296 (1995); Giovannangeli *et al. Biochemistry* 35:10539-10548 (1996); Chan and Glazer *J. Mol. Medicine (Berlin)* 75: 267-282 (1997)) and ribozymes (Zhao and Pick, *Nature* 365:448-451 (1993); Eastham and Ahlering, *J. Urology* 156:1186-1188 (1996); Sokol and Murray, *Transgenic Res.* 5:363-371 (1996); Sun *et al., Mol. Biotechnology* 7:241-251 (1997); and Haseloff *et al., Nature*, 334:585-591 (1988)).

Modification of endogenous *SNF1* or *SNF4* genes can also be used to inhibit expression. Methods for introducing genetic mutations described above can also be used to select for plants with decreased expression of genes of the invention.

Other means for inhibiting polynucleotide or polypeptide activity can also be used. Activity of polynucleotides of the invention may be modulated by eliminating the proteins that are required for cell-specific expression of such polynucleotides. Thus, expression of regulatory proteins and/or the sequences that control gene (e.g. *LeSNF4* or *LeSNF1*) expression can be modulated using the methods described here.

Purification of polypeptides

Naturally occurring or recombinant polypeptides of the invention can be purified for use in functional assays. Naturally occurring polypeptides can be purified, *e.g.*, from plant tissue and any other source of the desired polypeptide. Recombinant polypeptides can be purified from any suitable expression system.

The polypeptides of the invention may be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate; column chromatography, immunopurification methods, and others (see, *e.g.*, Scopes, *Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al., supra;* and Sambrook *et al., supra*).

A number of procedures can be employed when recombinant polypeptides are being purified. For example, proteins having established molecular adhesion properties (*e.g.* epitope tags, histidine tags and the like) can be reversibly fused to polypeptides of the invention. With the appropriate ligand, the such polypeptides can be selectively adsorbed to a purification column and then freed from the column in a relatively pure form.

Isolation of nucleic acids of the invention

Generally, the nomenclature and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturer's specifications. These techniques and various other techniques are generally performed according to Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989) or Current Protocols in Molecular Biology Volumes 1-3, John Wiley & Sons, Inc. (1994-1998).

The isolation of nucleic acids of the invention may be accomplished by a number of techniques. For instance, oligonucleotide probes based on the sequences disclosed here can be used to identify the desired gene in a cDNA or genomic DNA library. To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation, e.g. using restriction endonucleases, and are ligated with vector DNA to form concatemers that can be packaged into the appropriate vector. To prepare a cDNA library, mRNA is isolated from the desired organ, such as leaves, and a cDNA library that contains a gene transcript of the invention is prepared from the mRNA. Alternatively, cDNA may be prepared from mRNA extracted from other tissues in which genes of the invention or homologs are expressed.

The cDNA or genomic library can then be screened using a probe based upon the sequence of a cloned gene of the invention as disclosed here. Probes may be used to hybridize with genomic DNA or cDNA sequences to isolate homologous genes in the same or different plant species. Alternatively, antibodies raised against a polypeptide of the invention can be used to screen an mRNA expression library.

Alternatively, the nucleic acids of interest can be amplified from nucleic acid samples using amplification techniques. For instance, polymerase chain reaction (PCR) technology can be used to amplify the sequences of genes of the invention directly from genomic DNA, from cDNA, from genomic libraries or cDNA libraries. PCR and other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, for nucleic acid sequencing, or for other purposes. For a general overview of PCR, see *PCR Protocols: A Guide to Methods and Applications*. (Innis, M, Gelfand, D., Sninsky, J. and White, T.,

eds.), *Academic Press*, San Diego (1990). Appropriate primers and probes for identifying sequences of the invention from plant tissues are generated from comparisons of the sequences provided here (*e.g.* SEQ ID NO: 1, SEQ ID NO:3, etc.).

Polynucleotides may also be synthesized by well-known techniques as described in the technical literature. See, e.g., Carruthers *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* 47:411-418 (1982), and Adams *et al.*, *J. Am. Chem. Soc.* 105:661 (1983). Double stranded DNA fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Preparation of recombinant vectors

To use isolated sequences in the above techniques, recombinant DNA vectors suitable for transformation of plant cells are prepared. Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. See, for example, Weising et al. *Ann. Rev. Genet.* 22:421-477 (1988). A DNA sequence coding for the desired polypeptide, for example a cDNA sequence encoding a full length protein, will preferably be combined with transcriptional and translational initiation regulatory sequences which will direct the transcription of the sequence from the gene in the intended tissues of the transformed plant.

For example, for overexpression, a plant promoter fragment may be employed which will direct expression of the gene in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens*, and other transcription initiation regions from various plant genes known to those of skill. Constitutive promoters and regulatory elements can also be isolated from genes that are expressed constitutively or at least expressed in most if not all tissues of a plant. Such genes include, for example, *ACT11* from *Arabidopsis* (Huang *et al. Plant Mol. Biol.* 33:125-139 (1996)), *Cat3* from *Arabidopsis* (GenBank No. U43147, Zhong *et al.*, *Mol. Gen. Genet.* 251:196-203 (1996)), the gene encoding stearoyl-acyl carrier protein desaturase from *Brassica napus* (Genbank No. X74782, Solocombe *et al. Plant Physiol.* 104:1167-1176 (1994)), *GPc1* from maize (GenBank No.

X15596, Martinez *et al. J. Mol. Biol* 208:551-565 (1989)), and *Gpc2* from maize (GenBank No. U45855, Manjunath *et al.*, *Plant Mol. Biol.* 33:97-112 (1997)).

Alternatively, the plant promoter may direct expression of a nucleic acid of the invention in a specific tissue, organ or cell type (*i.e.* tissue-specific promoters) or may be otherwise under more precise environmental or developmental control (*i.e.* inducible promoters). Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic conditions, elevated temperature, the presence of light, or application of chemicals/hormones (such promoters can be used, for example, in the chemical induction of antisense *SNF4* sequences for breaking seed dormancy). Tissue-specific promoters may only promote transcription within a certain time frame of developmental stage within that tissue. Other tissue specific promoters may be active throughout the life cycle of a particular tissue. One of skill will recognize that a tissue-specific promoter may drive expression of operably linked sequences in tissues other than the target tissue. Thus, as used herein a tissue-specific promoter is one that drives expression preferentially in the target tissue or cell type, but may also lead to some expression in other tissues as well.

A number of tissue-specific promoters can be used in the invention. For instance, promoters that direct expression of nucleic acids in leaves, roots or flowers are useful for enhancing resistance to pathogens that infect those organs. For example, seed-specific promoters (*e.g.*, promoters from seed storage protein genes) can be used to direct expression of the polynucleotides of the invention and thereby enhance sink activity and starch or lipid accumulation in seeds. Alternatively, fruit specific promoters can be used to direct expression in fruit and thereby enhance sugar accumulation in fruit. For expression of a polynucleotide of the invention in the aerial vegetative organs of a plant, photosynthetic organ-specific promoters, such as the *RBCS* promoter (Khoudi, *et al.*, *Gene* 197:343, 1997), can be used. Root-specific expression of polynucleotides of the invention can be achieved under the control of the root-specific *ANR1* promoter (Zhang & Forde, *Science*, 279:407, 1998). Any strong, constitutive promoters, such as the CaMV *35S* promoter, can be used for the expression of polynucleotides of the invention throughout the plant.

Another example of a promoter useful in the present invention is the promoter of the *SNF4* gene provided in SEQ ID NO: 3 (residues 1-1097). One of skill that the variants of this promoter sequence can also be used. For example, the promoter can be less than full length (*e.g.* fragments of 500 to about 1000 nucleotides in length)

and still provide suitable expression levels. This promoter is useful because it responds to diverse stresses (*e.g.*, heat drought, cold) and to abscisic acid. The *SNF4* promoters of the invention can also be used to drive expression of heterologous nucleic acid sequences, whose expression would be advantageous under stress conditions. Examples of such nucleic acids include genes that control insect pests or pathogens (bacteria and fungi), such as *Bacillus thuringiensis* toxin, viral coat proteins, chitinases, phytoalexins, and the like. Genes that confer resistance to herbicides include, for example, genes encoding acetohydroxy acid synthase, phosphotransferases. Genes encoding glutathione reductase and superoxide dismutase are useful to confer resistance to fungal toxins and oxidative stress in cold, salinity, drought and wounding.If proper polypeptide expression is desired, a polyadenylation region at the 3'-end of the coding region should be included. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA.

The vector comprising the sequences (*e.g.*, promoters or coding regions) from genes of the invention will typically comprise a marker gene that confers a selectable phenotype on plant cells. For example, the marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or Basta.

Production of transgenic plants

DNA constructs of the invention may be introduced into the genome of the desired plant host by a variety of conventional techniques. For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using ballistic methods, such as DNA particle bombardment.

Microinjection techniques are known in the art and well described in the scientific and patent literature. The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski *et al. EMBO. J.* 3:2717-2722 (1984). Electroporation techniques are described in Fromm *et al. Proc. Natl. Acad. Sci. USA* 82:5824 (1985). Ballistic transformation techniques are described in Klein *et al. Nature* 327:70-73 (1987).

Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host

vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria. *Agrobacterium tumefaciens*-mediated transformation techniques, including disarming and use of binary vectors, are well described in the scientific literature. See, for example Horsch *et al. Science* 233:496-498 (1984), and Fraley *et al. Proc. Natl. Acad. Sci. USA* 80:4803 (1983) and *Gene Transfer to Plants*, Potrykus, ed. (Springer-Verlag, Berlin 1995).

Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype such as increased seed mass. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker that has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans et al., *Protoplasts Isolation and Culture, Handbook of Plant Cell Culture*, pp. 124-176, MacMillilan Publishing Company, New York, 1983; and Binding, *Regeneration of Plants, Plant Protoplasts*, pp. 21-73, CRC Press, Boca Raton, 1985. Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee et al. *Ann. Rev. of Plant Phys.* 38:467-486 (1987).

The nucleic acids of the invention can be used to confer desired traits on essentially any plant. Thus, the invention has use over a broad range of plants, including species from the genera *Anacardium, Arachis, Asparagus, Atropa, Avena, Brassica, Citrus, Citrullus, Capsicum, Carthamus, Cocos, Coffea, Cucumis, Cucurbita, Daucus, Elaeis, Fragaria, Glycine, Gossypium, Helianthus, Heterocallis, Hordeum, Hyoscyamus, Lactuca, Linum, Lolium, Lupinus, Lycopersicon, Malus, Manihot, Majorana, Medicago, Nicotiana, Olea, Oryza, Panieum, Pannesetum, Persea, Phaseolus, Pistachia, Pisum, Pyrus, Prunus, Raphanus, Ricinus, Secale, Senecio, Sinapis, Solanum, Sorghum, Theobromus, Trigonella, Triticum, Vicia, Vitis, Vigna,* and *Zea*.

One of skill will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

Using known procedures one of skill can screen for plants of the invention by detecting the increase or decrease of mRNA or protein of the invention in transgenic

plants. Means for detecting and quantitating mRNAs or proteins are well known in the art.

Plants with enhanced resistance to stress conditions can be selected in many ways. One of ordinary skill in the art will recognize that the following methods are but a few of the possibilities. One method of selecting plants with enhanced resistance is to determine resistance of a plant to a specific plant stress condition such as heat, cold, or nutritional deprivation. Other possible stress conditions include, but are not limited to, chemicals, metabolic changes (see, *e.g.*, Agrios, *Plant Pathology* (Academic Press, San Diego, CA) (1988)). One of skill in the art will recognize that resistance responses of plants vary depending on many factors, including what particular stress condition or plant is used. Generally, enhanced resistance is measured by the reduction or elimination of stress symptoms when compared to a control plant.

The following Examples are offered by way of illustration, not limitation.

EXAMPLES

Example 1

This example shows the isolation of the *LeSNF4* and *LeSNF1* cDNAs from tomato genomic library.

A partial cDNA was initially isolated by differential cDNA display corresponding to a mRNA that was down-regulated by gibberellin (GA) in association with tomato seed germination, and subsequently obtained cDNA and gDNA sequence from library screening. Differential Display (DCD) was performed according to known techniques.

Southern Blots of tomato genomic DNA suggested that the gene occurs only once in the genome. The gene encodes a 373 amino acid protein with a predicted molecular weight of 41 kD. A comparison of the amino acid sequence to yeast SNF4, mammalian γ-AMPK, and *Phaseolus vulgaris* L. Pv42 predicted protein is shown in Figure 1. The nucleic acid sequence and the encoded protein sequence of *LeSNF4* are shown in SEQ ID NO: 1 and SEQ ID NO: 2. The genomic sequence is shown in SEQ ID NO: 3.

The SNF4 and γ-AMPK proteins contain four repeats of a CBS motif identified from the cystathionine-β-synthase protein. Bateman, A., *Trends Biochem. Sci.* 22:12-13 (1997). This motif appears three times in the predicted tomato amino acid sequence, two near the C-terminal end and a third near the N-terminal end. The

intervening region where a fourth CBS domain is present in SNF4 and γ-AMPK is not identified as such in *LeSNF4*; however, this region is very highly conserved between tomato and bean, suggesting a role in protein function. The newly isolated gene is therefore termed the tomato gene *LeSNF4 (Lycopersicon esculentum SNF4)*.

Similarly, LeSNF1 gene was isolated from tomato, and the predicted protein has 85% identity and 90% similarity to the tobacco NPK5 protein. The nucleic acid sequence and the encoded protein sequence of LeSNF1 are shown in SEQ ID NO: 4 and SEQ ID NO: 5.

Example 2

This example shows the LeSNF4 gene is a functional homolog of the yeast SNF4 gene.

To test whether LeSNF4 is a functional homolog of the yeast SNF4, cDNA coding for the C-terminal 320 amino acids and the full length 373 amino acids of LeSNF4 (see Fig. 1) were independently transformed into a yeast line containing a deletion in the SNF4 gene (Δ-SNF4) according to standard techniques. The partial cDNA was used since this cDNA encodes a protein that most closely complements the yeast SNF4 protein, in both size and homology. The Δ-SNF4 mutants can grow on glucose, but not on sucrose or other sugars as the sole carbon source, since SNF4 protein is required to activate SNF1 kinase, which in turn derepresses invertase and other genes required to metabolize sucrose and other sugars. Schuller, H. and Entian, K., *Gene* 67:247 (1988); Woods, A., et al., *J. Biol. Chem.* 269:19509 (1994).

As expected, neither the Δ-SNF4 mutant nor the mutant cells transformed with the empty vector grew on medium containing sucrose as the sole carbon source. However, cells transformed with the partial or full length *LeSNF4* grew on sucrose-containing medium, demonstrating functional complementation of the SNF4 mutation. Similar results were obtained when galactose was used instead of sucrose as the sole carbon source. Southern hybridization of DNA from the various transformed lines confirmed that the *LeSNF4* cDNA was present in the transformed lines that exhibited growth on sucrose. Therefore, the results of this example show that both the full length 373 or partial 320 amino acids of LeSNF4 are able to functionally substitute for the 322 amino acid yeast SNF4 protein in the glucose repression system, confirming and extending the cross-kingdom parallels in this family of kinases.

Example 3

This example demonstrates that the expression of LeSNF4 and LeSNF1 in plants is affected by environmental and nutritional conditions.

In yeast, transcription of *SNF1* and *SNF4* is not affected by glucose, and mRNA amounts do not change markedly in response to environmental or nutritional conditions (Celenza and Carlson, *Mol. Cell Biol.* 4:54-60 (1984)). Similarly, some variations of amounts of AMPK mRNAs occur between organs, but differences are not great (Gao, *et al.*, *J. Biol. Chem.* 271:8675-8681 (1996); Mitchelhill, *et al.*, *J. Biol. Chem.* 269:2361-2364). Thus, it has been thought that expression of these genes is essentially constitutive with regulation being primarily biochemical through phosphorylation and dephosphorylation.

With respect to plant SnRKs, a recent review concluded that "little is known about regulation of the plant SNF1-related kinases *in vitro*, and almost nothing is known about their regulation *in vivo*". (Hardie, D., *et al.*, *Annu. Rev. Biochem.* 67:821-55 (1998)). Since *LeSNF4* was originally identified based on its differential expression during germination and exposure to GA, suggesting hormonal and/or developmental regulation of transcription, the mRNA expression patterns of *LeSNF1* and *LeSNF4* were further investigated.

LeSNF4 and *LeSNF1* transcripts were examined in wild type tomato (cv. Moneymaker) seeds under a range of conditions that influence germination. *LeSNF4* mRNA is abundant in dry seeds and those seeds that have not completed germination after 48 hours; but disappears completely in germinated seeds. *LeSNF1* transcripts were also abundant in dry seeds and seeds that had not germinated after 48 hours imbibition, but persisted in germinated seeds. Imbibing seeds in ABA, 1.2 MPa PEG osmotic solution, or under far-red irradiation, all of which inhibit radicle emergence, maintain the *LeSNF4* mRNA at high levels.

In contrast, *LeSNF1* transcript levels were not maintained by exogenous ABA or osmoticum and only partly by far-red illumination. *LeSNF4* mRNA was abundant in naturally dormant seeds that had not germinated after 14 days imbibition on water and in those seeds that failed to germinated when transferred to GA, but disappeared with 48 hours in those seeds that germinated when transferred to GA; *LeSNF1* was also present in dormant seeds but persisted on subsequent treatment with GA, irrespective of germination capacity. In each case, *LeSNF4* mRNA is present under

conditions where radicle emergence does not occur and disappears in seeds that complete germination; whereas *LeSNF1* transcripts were largely unaffected by different treatments.

The SNF1/AMPK complex has been suggested to be a stress response system (Hardie, *supra.*) In addition, many genes expressed during late embryogenesis are also expressed in vegetative tissues during periods of physiological water stress or in response to ABA. Therefore, the expression of *LeSNF4* and *LeSNF1* was examined in seedling leaves subjected to various forms of water stress. A single foliar spray of ABA induced *LeSNF4* expression within 6 hours; subsequently mRNA levels decreased and had disappeared by 24 hours. *LeSNF4* was abundantly and rapidly expressed by excision and dehydration, transiently during cold treatment, and to a lesser extent during the heat stress. In contrast, none of the imposed conditions significantly affected abundance of *LeSNF1* mRNA .

It is interesting to note that the *LeSNF4* expression is rapidly and dramatically up-regulated in response to physiological water stress and exogenous ABA, while the *LeSNF1* is largely constitutively expressed and unaffected by exogenous hormones. Similarly there is little evidence for transcriptional regulation of the SNF1 homologs in potato and tobacco, and it has been suggested that SnRKs may have a function in basic physiological processes in plant cells. Krapp, *et al., Plant J.* 3:817-828 (1993); Murnaka, *et al., supra.*).

Based on the results presented here SNF1-related protein kinases are involved in multiple signal transduction and/or regulation pathways, while only some of these functions may require and/or be regulated by SNF4-like proteins. For example, the tobacco and barley SNF1-related kinases are able to phosphorylate HMG-CoA reductase without SNF4 protein, at least *in vitro* . Barker, *et al., Plant Physiol.* 112:1141-1149 (1996); Muranaka, *et al., Plant Cell Physiol.* (Suppl) 38:02 (1997). On the other hand, SNF4 proteins may be required to react to any stress that alters cellular energy status or sugar supply or requires alteration in sugar partitioning.

By analogy with the yeast system, plant SNF1-related protein kinases play a role in sugar sensing. Jang and Sheen, *supra.*; Smeekens and Rook, *Plant Physiol.* 115:7-13 (1997)). Sugars control the expression of many plant genes, and thereby influence photosynthesis, metabolic processes and developmental transitions throughout the plant life cycle. At least three pathways have been identified for sugar sensing in plants, and SnRKs have been implicated as downstream factors in signal transduction. Protein phosphorylation/dephosphorylation is involved in diverse signaling pathways

throughout plant development, including those in developing and germinating seeds (Halford and Hardie *Plant Mol. Biol.* 37:735-748 (1998); Geogatsos, and Fisentzides, *Protein Phosphorylation in Plants* (Clarendon) 141:152; Walker-Simmons, *Seed Sci. Res.* 8:193-200 (1998)) and an intriguing hypothesis has been put forward invoking this reversible process as a time-keeping mechanism in seed dormancy (Trewavas, A., *BioEssays*, 6:87-92 (1998)).

Glucose is generally absent or present at low levels in mature seeds, while sucrose accumulates to high concentrations. Amuti, K. and Pollard, C., *Phytochem.* 16:529-532 (1977). There is strong evidence that soluble sugars, particularly sucrose and oligosaccharides such as raffinose and stachyose, are involved in desiccation tolerance and longevity in seeds, and in vegetative water and cold stress. Therefore, under these conditions carbohydrate metabolism is directed to conserve and/or accumulate these sugars, rather than use them as an immediate energy source. Recently direct evidence for the role of sugars in seed development has begun to emerge. During seed development, the change from cell division to cell differentiation is accompanied by a reduction in hexose concentration and increase in sucrose levels (Borisjuk, et al., *Plant J.* 15:583-591 (1998)). In order to investigate the role of sugar status and carbon partitioning in developing seeds, Weber, *et al.*, *Plant J.* 16:163-172 (1998), expressed a yeast invertase gene in maturing embryos of *Vicia narbonensis*. The transgenic cotyledons showed a marked reduction in sucrose and elevated hexose levels causing a fall in starch and storage protein accumulation. In addition genes of sucrose and starch metabolism were specifically down regulated. These results suggest that a sucrose specific sensing mechanism in the storage cells senses sucrose and initiates storage associated differentiation.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference for all purposes.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising a polynucleotide sequence encoding a plant SNF4 polypeptide, wherein the polypeptide comprises an amino acid sequence that has at least about 70% identity to SEQ ID NO: 3.

2. The nucleic acid molecule of claim 1, wherein the polynucleotide sequence specifically hybridizes to SEQ ID NO: 1 or its complement, or SEQ ID NO: 2 or its complement.

3. The nucleic acid molecule of claim 1, wherein the SNF4 polypeptide is as shown in SEQ ID NO: 3.

4. The nucleic acid molecule of claim 1, wherein the polynucleotide sequence is as shown in SEQ ID NO: 1.

5. A recombinant expression vector comprising the polynucleotide sequence of claim 1.

6. The recombinant expression vector of claim 5, further comprising a promoter operably linked to the polynucleotide sequence.

7. The recombinant expression vector of claim 6, wherein the promoter is a constitutive promoter.

8. The recombinant expression vector of claim 6, wherein the promoter is an inducible promoter.

9. A host cell transformed with the recombinant expression vector of claim 5.

10. A transgenic plant comprising a recombinant expression cassette comprising a promoter operably linked to a polynucleotide sequence of claim 1.

11. The transgenic plant of claim 10, wherein the plant is tomato.

12. The transgenic plant of claim 10, wherein the polynucleotide sequence specifically hybridizes to SEQ ID NO: 1 or its complement, or SEQ ID NO: 2 or its complement.

13. The transgenic plant of claim 10, wherein the polypeptide is as shown in SEQ ID NO: 3..

14 A method of modulating sugar metabolism in a plant, the method comprising

a) introducing into the plant a recombinant expression vector comprising a promoter operably linked to a polynucleotide sequence encoding a plant SNF4 polypeptide, wherein the polypeptide comprises an amino acid sequence that has at least about 70% identity to SEQ ID NO: 3; and

b) selecting a plant with modulated sugar metabolism.

15. The method of claim 14, wherein the polynucleotide specifically hybridizes to SEQ ID NO: 1 or its complement, or SEQ ID NO: 2 or its complement.

16. The method of claim 14, wherein the polypeptide is as shown in SEQ ID NO: 3.

17. The method of claim 14, wherein the modulation of sugar metabolism is associated with a response to stress conditions.

18. An isolated nucleic acid molecule comprising a polynucleotide sequence encoding a plant SNF1 polypeptide, wherein the polynucleotide sequence specifically hybridizes to SEQ ID NO: 4 or its complement.

19. The nucleic acid molecule of claim 1, wherein the polynucleotide sequence is as shown in SEQ ID NO: 4.

20. A recombinant expression vector comprising the polynucleotide sequence of claim 18.

21. The recombinant expression vector of claim 20 further comprising a promoter operably linked to the polynucleotide sequence.

22. The recombinant expression vector of claim 21 wherein the promoter is a constitutive promoter.

23. The recombinant expression vector of claim 21, wherein the promoter is an inducible promoter.

24. A host cell transformed with the recombinant expression vector of claim 20.

25. A transgenic plant comprising a recombinant expression cassette comprising a promoter operably linked to a polynucleotide sequence of claim 18.

26. The transgenic plant of claim 25, wherein the plant is tomato.

27. The transgenic plant of claim 25, wherein the polypeptide is as shown in SEQ ID NO: 4..

28. A method of modulating sugar metabolism in a plant, the method comprising

a) introducing into the plant a recombinant expression vector comprising a promoter operably linked to a polynucleotide sequence encoding a plant SNF1 polypeptide, wherein the polynucleotide sequence specifically hybridizes to SEQ ID NO: 4 or its complement and

b) selecting a plant with modulated sugar metabolism.

29. The method of claim 28, wherein the polypeptide is as shown in SEQ ID NO: 4.

30. The method of claim 28, wherein the modulation of sugar metabolism is associated with a response to stress conditions.

31. An isolated nucleic acid molecule comprising a promoter sequence operably linked to a heterologous sequence, wherein the promoter sequence comprises a sequence at least about 70% identical to a sequence from residue 1 to about 1097 of SEQ ID NO: 2.

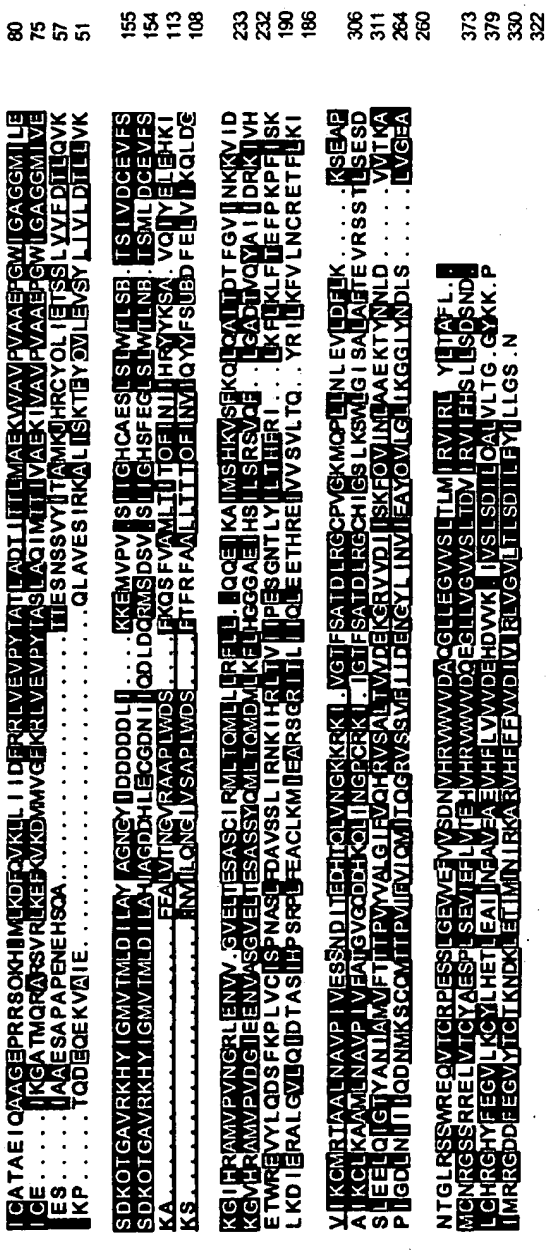


FIG. 1

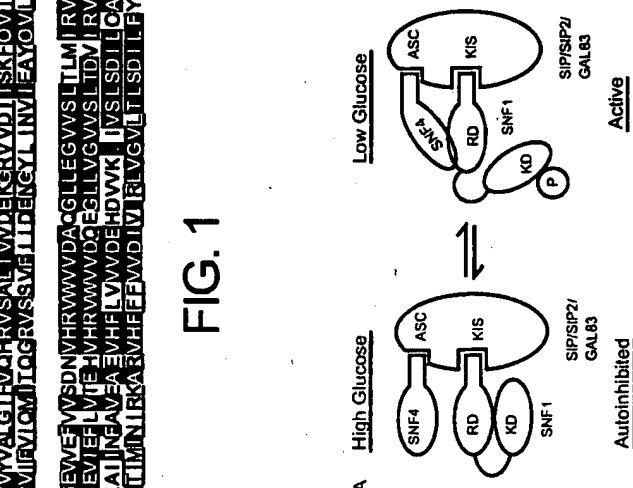

A
High Glucose
Low Glucose
SNF4
ASC
RD
KIS
KD
SNF1
SIP/SIP2/
GAL83
Autoinhibited
Active
P

FIG. 2

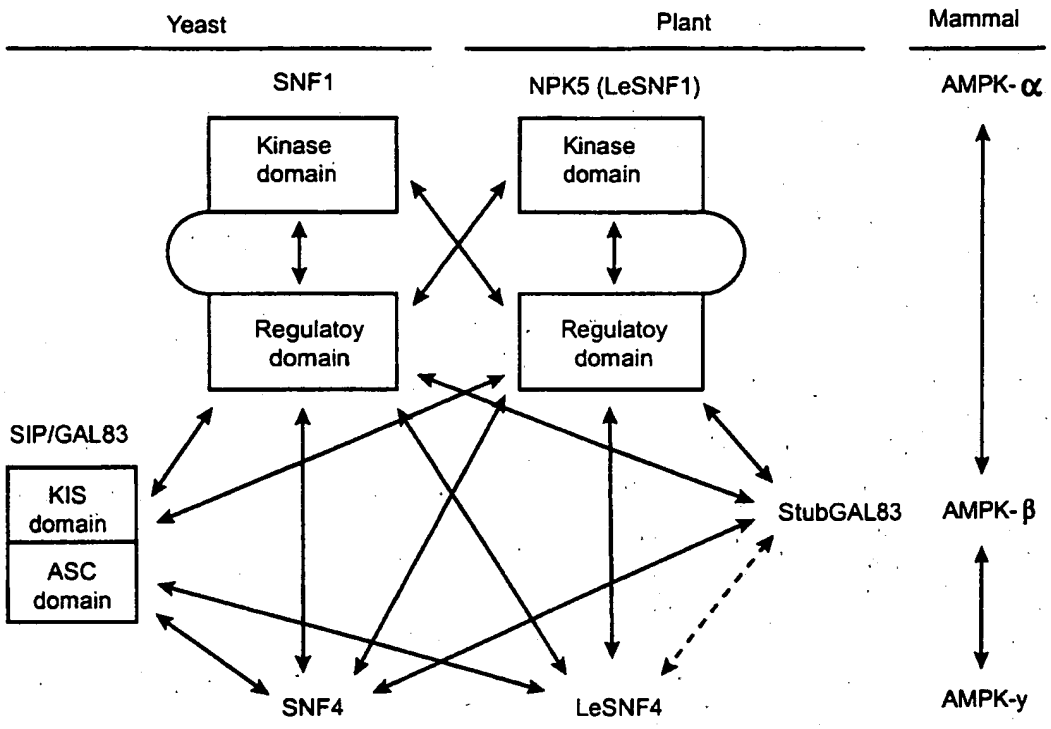

Yeast
Plant
Mammal
SNF1
NPK5 (LeSNF1)
AMPK-α
Kinase domain
Kinase domain
Regulatoy domain
Regulatoy domain
SIP/GAL83
KIS domain
ASC domain
StubGAL83
AMPK-β
SNF4
LeSNF4
AMPK-y

FIG. 2B

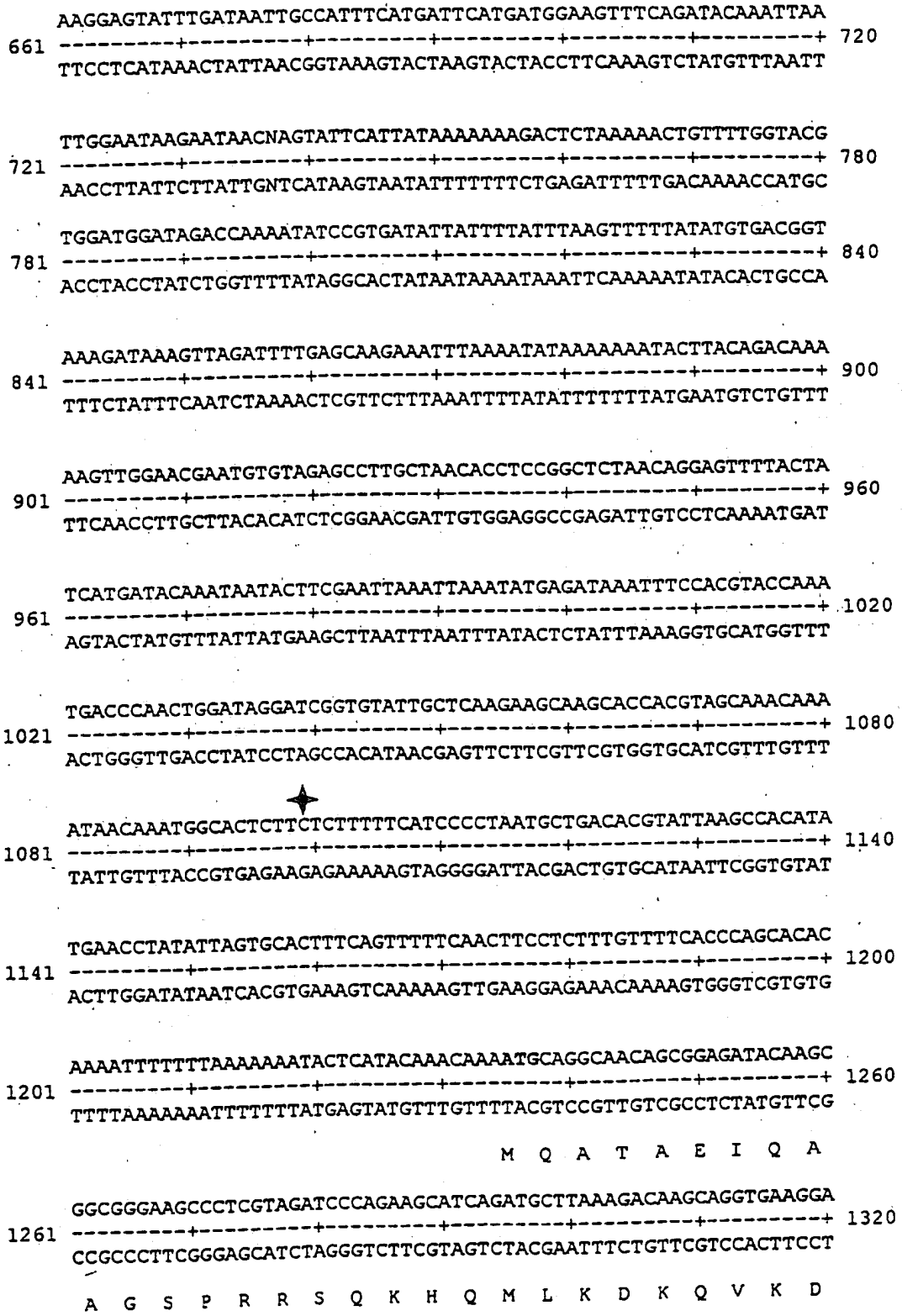
LeSNF4 (Sucrose nonfermenting (SNF) yeast homolog) gene from tomato seed (Expressed all the time when seeds don not germinate), DNA 1262 bp, expressed protein 373 aa.

CTCTTTGTTTCACCCAGCACACAAAATTTTAAAAAAAAAATACTCATACAAACAAA
ATGCAGGCAACAGCGGAGATACAAGCGGCGGGAAGCCCTCGTAGATCTCAGAAGCA
TCAGATGCTTAAAGACAAGCAGGTGAAGGATCTAATTATTGATAAAAGGAGACTTG
TGGAGGTTCCGTATACAGCCACGCTGGCAGATACAATAAACACTCTGATGGCTAAC
AAGGTGGTGGCGGTTCCGGTGGCTGCACCGCCTGGGCACTGGATTGGCGCCGGCGG
TTCTATGATTTTGGAATCTGATAAACAGACGGGTGCTGTACGAAAACATTATATAG
GGATGGTAACTATGCTTGATATTCTCGCATATATTGCTGGAAACGGTTATCGTGAT
GATGATGATGATCTTACGAAAAAGATGATGGTTCCTGTTTCTTCGATTATTGGGCA
TTGTCTTGAAAGTCTTAGTTTGTGGACCCTCAGCCCTAACACAAGTATTGTGGATT
GTATGGAAGTTTTCAGCAAAGGCATACATCGAGCCATGGTACCAGTGAATGGACGA
TTAGAAAATGTAGTTGGCGTTGAGCTCACCGAGTCAGCGTCATGTTACCGAATGCT
AACACAAATGGATCTGCTTAGGTTTTTGAATGACCAGCAGGAGCTTAAAGCGATCA
TGTCGCACAAGGTCTCGGATAAACAACTCCAAGCAATCACAGACACTGTTTTCGGT
GTGACTAATAAGGCGAAAGTTATCGATGTGATCAAATGCATGAGAACAGCTTCACT
AAATGCAGTACCAATTGTGGAGTCATCCAATGACATAACAGAAGATCATACTCAGC
TTGTGAATGGGAAAAAGAGGAAGATTGTAGGAACATTTTCAGCAACGGACTTGAGA
GGCTGTCCTGTATCGAAAATGCAGCCTCTATTGAACCTAGAGGTCCTCGATTTCTT
GAAAATGCTGTCGGGAGCTCCTAATACCGGGCTGAGATCTTCATGGAGGGAACAAG
TGACATGCCGCCCCGAATCGTCACTCGGGGAAGTGGTAGAGAAAGTTGTTTCAGAC
AATGTGCATCGTGTTTGGGTGGTGGATGAACAAGGCTTGCTGGAAGGAGTTGTATC
CCTAACTGACATGATAAGAGTCATCAGACTCTGGTATCTTACTGAGTTTTTGCAGT
GATGTGTAGTTGTTGTACACACTCTTTTGCACCCGTTTTGTTCTGGTTGGTGTTTT
GGTGTATAAATGAAATGCAATCTGTTTATC

SEQUENCE I.D. NO. 2 & 3

LeSNF4 genomic clone with intron and coding region.
The cDNA/mRNA starts at the ✦. The promoter is from base 1 to 1097...
The DNA used for transformation is from 1 to 1235 (i.e. upto –1 of ATG start codon).

```
      AAGCGGTCACATTTTTTAAGAAGATGGTTATCTCAATCAACATCTTGGATACCAAAAAAG
    1 ---------+---------+---------+---------+---------+---------+ 60
      TTCGCCAGTGTAAAAAATTCTTCTACCAATAGAGTTAGTTGTAGAACCTATGGTTTTTTC

      AAAGCCTTAGCCTAACCTAGACAATCCCTTCACTTTGCTACCGGGTGCGTCTGTCTAACT
   61 ---------+---------+---------+---------+---------+---------+ 120
      TTTCGGAATCGGATTGGATCTGTTAGGGAAGTGAAACGATGGCCCACGCAGACAGATTGA

      TTGAATTAACTAGGGCTGAGAAACTGGTCAGTTAAAGTTAAATGGTGTCTTATATACTGT
  121 ---------+---------+---------+---------+---------+---------+ 180
      AACTTAATTGATCCCGACTCTTTGACCAGTCAATTTCAATTTACCACAGAATATATGACA

      TGAATATGAGGATCTTTAGATATATTCTTTATTTGTTTATTGAATAGGTTGTCAATCCGA
  181 ---------+---------+---------+---------+---------+---------+ 240
      ACTTATACTCCTAGAAATCTATATAAGAAATAAACAAATAACTTATCCAACAGTTAGGCT

      AGTTGTTACTGCTAATTCAATTATTATTATGGCTTTGGATGCTTACCCAATTACTCATAA
  241 ---------+---------+---------+---------+---------+---------+ 300
      TCAACAATGACGATTAAGTTAATAATAATACCGAAACCTACGAATGGGTTAATGAGTATT

      TCTCTTCTTCTTGCAGATGATAGCAGCTAATTTCTTGTTCATAGAGAAATATACATCATA
  301 ---------+---------+---------+---------+---------+---------+ 360
      AGAGAAGAAGAACGTCTACTATCGTCGATTAAAGAACAAGTATCTCTTTATATGTAGTAT

      ATTCCATACGCCCTCTTTCTGTTTCTTTTACCTTCTAATCCACTAGACAAAGTGCTAGGG
  361 ---------+---------+---------+---------+---------+---------+ 420
      TAAGGTATGCGGGAGAAAGACAAAGAAAATGGAAGATTAGGTGATCTGTTTCACGATCCC

      TGGACCTAAACGAGGCTCCCTCCGTCCTATTTTACGTGATACTTTTTAAATTTTAAATAA
  421 ---------+---------+---------+---------+---------+---------+ 480
      ACCTGGATTTGCTCCGAGGGAGGCAGGATAAAATGCACTATGAAAAATTTAAAATTTATT

      ATTAATTTTTTATCATAAATTTTTTATAAATATTTTAACATTTTAAATTATAATATATTT
  481 ---------+---------+---------+---------+---------+---------+ 540
      TAATTAAAAAATAGTATTTAAAAAATATTTATAAAATTGTAAAATTTAATATTATATAAA

      TGATTTATTATAATATTTACGTAATTTATAAATATATAAAATTTATTTATAAATTTTTAA
  541 ---------+---------+---------+---------+---------+---------+ 600
      ACTAAATAATATTATAAATGCATTAAATATTTATATATTTTAAATAAATATTTAAAAATT

      TTAAATTTAAATTATTTAACTCTAATTGAAAAATACTTAACTAACGATAAATGGAAAGAG
  601 ---------+---------+---------+---------+---------+---------+ 660
      AATTTAAATTTAATAAATTGAGATTAACTTTTTATGAATTGATTGCTATTTACCTTTCTC
```


AAGGAGTATTTGATAATTGCCATTTCATGATTCATGATGGAAGTTTCAGATACAAATTAA
661 ---------+---------+---------+---------+---------+---------+ 720
TTCCTCATAAACTATTAACGGTAAAGTACTAAGTACTACCTTCAAAGTCTATGTTTAATT
TTGGAATAAGAATAACNAGTATTCATTATAAAAAAAGACTCTAAAAACTGTTTTGGTACG
721 ---------+---------+---------+---------+---------+---------+ 780
AACCTTATTCTTATTGNTCATAAGTAATATTTTTTTCTGAGATTTTTGACAAAACCATGC
TGGATGGATAGACCAAAATATCCGTGATATTATTTTATTTAAGTTTTTATATGTGACGGT
781 ---------+---------+---------+---------+---------+---------+ 840
ACCTACCTATCTGGTTTTATAGGCACTATAATAAAATAAATTCAAAAATATACACTGCCA
AAAGATAAAGTTAGATTTTGAGCAAGAAATTTAAAATATAAAAAAAATACTTACAGACAAA
841 ---------+---------+---------+---------+---------+---------+ 900
TTTCTATTTCAATCTAAAACTCGTTCTTTAAATTTTATATTTTTTTATGAATGTCTGTTT
AAGTTGGAACGAATGTGTAGAGCCTTGCTAACACCTCCGGCTCTAACAGGAGTTTTACTA
901 ---------+---------+---------+---------+---------+---------+ 960
TTCAACCTTGCTTACACATCTCGGAACGATTGTGGAGGCCGAGATTGTCCTCAAAATGAT
TCATGATACAAATAATACTTCGAATTAAATTAAATATGAGATAAATTTCCACGTACCAAA
961 ---------+---------+---------+---------+---------+---------+ 1020
AGTACTATGTTTATTATGAAGCTTAATTTAATTTATACTCTATTTAAAGGTGCATGGTTT
TGACCCAACTGGATAGGATCGGTGTATTGCTCAAGAAGCAAGCACCACGTAGCAAACAAA
1021 ---------+---------+---------+---------+---------+---------+ 1080
ACTGGGTTGACCTATCCTAGCCACATAACGAGTTCTTCGTTCGTGGTGCATCGTTTGTTT
ATAACAAATGGCACTCTTCTCTTTTTCATCCCCTAATGCTGACACGTATTAAGCCACATA
1081 ---------+---------+---------+---------+---------+---------+ 1140
TATTGTTTACCGTGAGAAGAGAAAAAGTAGGGGATTACGACTGTGCATAATTCGGTGTAT
TGAACCTATATTAGTGCACTTTCAGTTTTTCAACTTCCTCTTTGTTTTCACCCAGCACAC
1141 ---------+---------+---------+---------+---------+---------+ 1200
ACTTGGATATAATCACGTGAAAGTCAAAAAGTTGAAGGAGAAACAAAAGTGGGTCGTGTG
AAAATTTTTTTAAAAAAATACTCATACAAACAAAATGCAGGCAACAGCGGAGATACAAGC
1201 ---------+---------+---------+---------+---------+---------+ 1260
TTTTAAAAAAATTTTTTTATGAGTATGTTTGTTTTACGTCCGTTGTCGCCTCTATGTTCG
M Q A T A E I Q A
GGCGGGAAGCCCTCGTAGATCCCAGAAGCATCAGATGCTTAAAGACAAGCAGGTGAAGGA
1261 ---------+---------+---------+---------+---------+---------+ 1320
CCGCCCTTCGGGAGCATCTAGGGTCTTCGTAGTCTACGAATTTCTGTTCGTCCACTTCCT
A G S P R R S Q K H Q M L K D K Q V K D

```
     TCTAATTATTGATAAAAGGAGACTTGTGGAGGTTCCGTATACAGCCACGCTGGCAGATAC
1321 ---------+---------+---------+---------+---------+---------+ 1380
     AGATTAATAACTATTTTCCTCTGAACACCTCCAAGGCATATGTCGGTGCGACCGTCTATG

      L  I  I  D  K  R  R  L  V  E  V  P  Y  T  A  T  L  A  D  T

     AATAAACACTCTGATGGCTAACAAGGTGGTGGCGGTTCCGGTGGCTGCACCGCCTGGGCA
1381 ---------+---------+---------+---------+---------+---------+ 1440
     TTATTTGTGAGACTACCGATTGTTCCACCACCGCCAAGGCCACCGACGTGGCGGACCCGT

      I  N  T  L  M  A  N  K  V  V  A  V  P  V  A  A  P  P  G  H

     CTGGATTGGCGCCGGCGGTTCTATGATTTTGGAATCTGATAAACAGACGGGTGCTGTACG
1441 ---------+---------+---------+---------+---------+---------+ 1500
     GACCTAACCGCGGCCGCCAAGATACTAAAACCTTAGACTATTTGTCTGCCCACGACATGC

      W  I  G  A  G  G  S  M  I  L  E  S  D  K  Q  T  G  A  V  R

     AAAACATTATATAGGGATGGTAACTATGCTTGATATTCTCGCATATATTGCTGGAAACGG
1501 ---------+---------+---------+---------+---------+---------+ 1560
     TTTTGTAATATATCCCTACCATTGATACGAACTATAAGAGCGTATATAACGACCTTTGCC

      K  H  Y  I  G  M  V  T  M  L  D  I  L  A  Y  I  A  G  N  G

     TTATCGTGATGATGATGATGATCTTACGAAAAAGATGATGGTTCCTGTTTCTTCGATTAT
1561 ---------+---------+---------+---------+---------+---------+ 1620
     AATAGCACTACTACTACTACTAGAATGCTTTTTCTACTACCAAGGACAAAGAAGCTAATA

b     Y  R  D  D  D  D  D  L  T  K  K  M  M  V  P  V  S  S  I  I

     TGGGCATTGTCTTGAAAGTCTTAGTTTGTGGACCCTCAGCCCTAACACAAGGTATGTAAC
1621 ---------+---------+---------+---------+---------+---------+ 1680
     ACCCGTAACAGAACTTTCAGAATCAAACACCTGGGAGTCGGGATTGTGTTCCATACATTG

b     G  H  C  L  E  S  L  S  L  W  T  L  S  P  N  T
     GAGGAATTGAGATCTCTTGGCCGCTTACAAAAATAAAAACTGATGTTCTCCTCATGGGTA
1681 ---------+---------+---------+---------+---------+---------+ 1740
     CTCCTTAACTCTAGAGAACCGGCGAATGTTTTTATTTTTGACTACAAGAGGAGTACCCAT

     AAAATGAAGAGGAGATCACAGAACTAAGAAAATTAAGAAATTATTATGAGTAACCTACAA
1741 ---------+---------+---------+---------+---------+---------+ 1800
     TTTTACTTCTCCTCTAGTGTCTTGATTCTTTTAATTCTTTAATAATACTCATTGGATGTT

     AATGAATCCTGTAGAAAGTAGTGGATGTTGTTAGCAAATACTTTTAATCTATCAAATTGG
1801 ---------+---------+---------+---------+---------+---------+ 1860
     TTACTTAGGACATCTTTCATCACCTACAACAATCGTTTATGAAAATTAGATAGTTTAACC

     AATCGGAGTAAGGATTTTTATAATTATGATTACTTCAATAAAGTCCTTGCTGCTATTTGT
1861 ---------+---------+---------+---------+---------+---------+ 1920
     TTAGCCTCATTCCTAAAAATATTAATACTAATGAAGTTATTTCAGGAACGACGATAAACA

     CAAATGAATTTTGGATATTCAAGGTATTATCTAATTTTGCAAATGTATCTTACGAATTGA
1921 ---------+---------+---------+---------+---------+---------+ 1980
```

```
    GTTTACTTAAAACCTATAAGTTCCATAATAGATTAAAACGTTTACATAGAATGCTTAACT

    TTCTTTATATGTGAAGTATTGTGGATTGTATGGAAGTTTTCAGCAAAGGCATACATCGAG
1981 ---------+---------+---------+---------+---------+---------+ 2040
    AAGAAATATACACTTCATAACACCTAACATACCTTCAAAAGTCGTTTCCGTATGTAGCTC

                  S  I  V  D  C  M  E  V  F  S  K  G  I  H  R  A

    CCATGGTACCAGTGAATGGACGATTAGAAAATGTAGTTGGCGTTGAGCTCACCGAGTCAG
2041 ---------+---------+---------+---------+---------+---------+ 2100
    GGTACCATGGTCACTTACCTGCTAATCTTTTACATCAACCGCAACTCGAGTGGCTCAGTC

      M  V  P  V  N  G  R  L  E  N  V  V  G  V  E  L  T  E  S  A

    CGTCATGTTACCGAATGCTAACACAAATGGATCTGCTTAGGTTTTTGAATGACCAGCAGG
2101 ---------+---------+---------+---------+---------+---------+ 2160
    GCAGTACAATGGCTTACGATTGTGTTTACCTAGACGAATCCAAAAACTTACTGGTCGTCC

      S  C  Y  R  M  L  T  Q  M  D  L  L  R  F  L  N  D  Q  Q  E

    AGCTTAAAGCGATCATGTCGCACAAGGTCTCGGATAAACAACTCCAAGCAATCACAGACA
2161 ---------+---------+---------+---------+---------+---------+ 2220
    TCGAATTTCGCTAGTACAGCGTGTTCCAGAGCCTATTTGTTGAGGTTCGTTAGTGTCTGT

      L  K  A  I  M  S  H  K  V  S  D  K  Q  L  Q  A  I  T  D  T

    CTGTTTTCGGTGTGACTAATAAGGCGAAAGTTATCGATGTGATCAAATGCATGAGAACAG
2221 ---------+---------+---------+---------+---------+---------+ 2280
    GACAAAAGCCACACTGATTATTCCGCTTTCAATAGCTACACTAGTTTACGTACTCTTGTC

      V  F  G  V  T  N  K  A  K  V  I  D  V  I  K  C  M  R  T  A

    CTTCACTAAATGCAGTACCAATTGTGGAGTCATCCAATGACATAACAGAAGATCATACTC
2281 ---------+---------+---------+---------+---------+---------+ 2340
    GAAGTGATTTACGTCATGGTTAACACCTCAGTAGGTTACTGTATTGTCTTCTAGTATGAG

      S  L  N  A  V  P  I  V  E  S  S  N  D  I  T  E  D  H  T  Q

    AGCTTGTGAATGTAAGTTAAAACATTTAGTCTCACTCGTTTGAATTTAATTTATATATAT
2341 ---------+---------+---------+---------+---------+---------+ 2400
    TCGAACACTTACATTCAATTTTGTAAATCAGAGTGAGCAAACTTAAATTAAATATATATA

      L  V  N

    ACCTCTTAACGATATCAGGATTATTGTTTTCTATGAAATATACAGGGGAAAAAGAGGAAG
2401 ---------+---------+---------+---------+---------+---------+ 2460
    TGGAGAATTGCTATAGTCCTAATAACAAAAGATACTTTATATGTCCCCTTTTTCTCCTTC

                                                G  K  K  R  K

    ATTGTAGGAACATTTTCAGCAACGGACTTGAGAGGCTGTCCTGTATCGAAAATGCAGCCT
2461 ---------+---------+---------+---------+---------+---------+ 2520
    TAACATCCTTGTAAAAGTCGTTGCCTGAACTCTCCGACAGGACATAGCTTTTACGTCGGA

    I  V  G  T  F  S  A  T  D  L  R  G  C  P  V  S  K  M  Q  P
```

```
    CTATTGAACCTAGAGGTCCTCGATTTCTTGAAAATGCTGTCGGGAGCTCCTAATACCGGG
2521 ---------+---------+---------+---------+---------+---------+ 2580
    GATAACTTGGATCTCCAGGAGCTAAAGAACTTTTACGACAGCCCTCGAGGATTATGGCCC

    L  L  N  L  E  V  L  D  F  L  K  M  L  S  G  A  P  N  T  G

    CTGAGATCTTCATGGAGGGAACAAGTGACATGCCGCCCCGAATCGTCACTCGGGGAAGTG
2581 ---------+---------+---------+---------+---------+---------+ 2640
    GACTCTAGAAGTACCTCCCTTGTTCACTGTACGGCGGGGCTTAGCAGTGAGCCCCTTCAC

    L  R  S  S  W  R  E  Q  V  T  C  R  P  E  S  S  L  G  E  V

    GTAGAGAAAGTTGTTTCAGACAATGTGCATCGTGTTTGGGTGGTGGATGAACAAGGCTTG
2641 ---------+---------+---------+---------+---------+---------+ 2700
    CATCTCTTTCAACAAAGTCTGTTACACGTAGCACAAACCCACCACCTACTTGTTCCGAAC

    V  E  K  V  V  S  D  N  V  H  R  V  W  V  V  D  E  Q  G  L

    CTGGAAGGAGTTGTATCCCTAACTGACATGATAAGAGTCATCAGACTCTGGTATCTTACT
2701 ---------+---------+---------+---------+---------+---------+ 2760
    GACCTTCCTCAACATAGGGATTGACTGTACTATTCTCAGTAGTCTGAGACCATAGAATGA

    L  E  G  V  V  S  L  T  D  M  I  R  V  I  R  L  W  Y  L  T

    GAGTTTTTGCAGTGATGTGTAGTTGTTGTACGCACTCTTTTGCACCCGTTTTGTTCTGGT
2761 ---------+---------+---------+---------+---------+---------+ 2820
    CTCAAAAACGTCACTACACATCAACAACATGCGTGAGAAAACGTGGGCAAAACAAGACCA

    E  F  L  Q  *

    TGGTGTTTTGGTGTATAAATGAAATGCAATCTGTTTATCAAATGATCAGTTCTCATTCTG
2821 ---------+---------+---------+---------+---------+---------+ 2880
    ACCACAAAACCACATATTTACTTTACGTTAGACAAATAGTTTACTAGTCAAGAGTAAGAC

    AAAATTTGTTTTGTGTCTTACACAAGCGACCCTAAAGGAAAACAATAGGTACTACTAGAT
2881 ---------+---------+---------+---------+---------+---------+ 2940
    TTTTAAACAAAACACAGAATGTGTTCGCTGGGATTTCCTTTTGTTATCCATGATGATCTA

    TGAAATTTTTCTACCATATCGCTTTCATATAATTATTAATGAACACTACTCTTATGTTCC
2941 ---------+---------+---------+---------+---------+---------+ 3000
    ACTTTAAAAAGATGGTATAGCGAAAGTATATTAATAATTACTTGTGATGAGAATACAAGG

    GGGGACAAACATAAAGCTT
3001 ---------+--------- 3019
    CCCCTGTTTGTATTTCGAA
```

LeSNF1 (Yeast SNF1 homolog) gene from tomato seed, DNA 1929 bp, predicted protein 514 aa.

GGCACGAGGTGGAAGGAAAACATCAAAGGAAAATGGACGGAACAGCAGTGCAGGGC
ACCAGCAGTGTTGACTCATTTTTACGGAACTATAAACTCGGGAAAACACTTGGCAT
TGGATCGTTCGGCAAAGTTAAAATAGCTGAACATACGTTAACAGGGCACAAAGTTG
CTGTCAAGATTCTTAATCGTCGAAAAATCAGGAATATGGACATGGAGGAGAAAGTC
CGTAGAGAAATCAAAATATTGAGATTGTTCATGCATCCTCATATTATACGGCTTTA
TGAGGTCATAGAGACACCATCAGATATATATGTTGTGATGGAGTATGTGAAATCTG
GCGAGTTATTTGATTACATTGTTGAGAAGGGCAGATTGCAGGAGGATGAAGCTCGT
AACTTTTTTCAGCAGATAATTTCTGGTGTGGAGTACTGCCATAGAAACATGGTGGT
TCATAGAGACCTTAAGCCTGAAAACCTCCTTCTGGACTCCAAATGGAATGTGAAGA
TCGCAGATTTTGGTTTGAGCAATATCATGCGCGATGGTCATTTTCTGAAGACAAGT
TGCGGAAGCCCAAACTATGCTGCCCCAGAGGTTATATCAGGTAAATTGTATGCTGG
CCCTGAGGTAGATGTATGGAGCTGTGGTGTTATTCTTTATGCTCTTCTCTGTGGCA
CCCTTCCGTTTGACGATGAAAACATACCCAATCTTTTTAAGAAAATAAAGGGTGGA
ATATATACTCTGCCCAGCCATTTATCAGCTGGTGCGAGGGATTTGATTCCGAGGAT
GCTTATAGTCGACCCAATGAAGCGAATGACTATTCCTGAGATTCGCCTGCACCCTT
GGTTCCAAGCTCATTTGCCACGCTATTTGGCCGTGCCTCCACCAGATACAACCCAA
CAAGCAAAGAAGATCGATGAAGAGATTCTTCAAGAGGTGGTTAAGATGGGATTTGA
CAGGAACAACCTTACTGAGTCTCTTCGCAATAGAGTTCAAAATGAGGGCACTGTTG
CATACTATCTGCTCCTGGACAATCGCCATCGTGTTTCCACTGGCTATCTTGGAGCT
GAATTTCAGGAGTCCATGGAATATGGTTACAACCGGATCAATTCTAATGAAACCGC
TGCTTCCCCTGTTGGTCAACGTTTCCCAGGAATAATGGATTATCAGCAAGCTGGTG
CAAGACAGTTCCCCATTGAAAGAAAATGGGCTCTTGGCCTCCAGTCTCGAGCGCAT
CCACGTGAAATAATGACTGAAGTTTTGAAAGCTCTGCAAGAACTGAATGTATGTTG
GAAAAGATTGGTCAGTATAACATGAAATGTCGATGGGTTCCTAGCTTACCTGGTCA
TCATGAAGGCATGGGTGTTAATTCCATGCATGGGAATCAGTTCTTTGGAGATGATT
CATCCATCATTGAGAATGATGGGGCCACAAAGTTAACAAATGTGGTCAAGTTTGAA
GTTCAGCTTTACAAAACCAGGGAGGAGAAGTACTTGCTTGACCTTCAGAGACTTCA
GGGTCCACAATTCCTCTTCCTGGATCTCTGTGCTGCTTTTCTTGCTCAGCTTCGAG
TACTTTAAAGTCTCCGAAATAAGGAGCTAAGTTGGAAAAAGCCCATGCTTGTATAT
AATTGGTATACCAGCTCAGTTACTGCATTTTGTCTTGTTAACAAATTCCACCCTGC
TTGGTCAGAGGTGCCTAGCAACTCTTTTTTTCTTTTGATTCGCTAGGAGATCTAGC
TCACTCTCTTTTTTAACGTTTATGGAATTTCAGTTACCTACAGTATCTACTTACAG
ATTGAACTGCAAGATGAGCGCGATGTCTGTCTGTGACCCATTCCCTCTTCTCCCCT
TATCGACGATCATTTGGAGTCCAACGAAGATTTTCTTGTGTCAAATTTGAAATGTT
TGTCTGAATAAAAACAAGTCCCAAC

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/19981

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 5/04, 15/29, 15/82; C07H 21/04; A01H 5/00; C12P 19/00
US CL : 800/284, 317.4; 536/23.6, 24.1; 435/320.1, 419

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/284, 317.4; 536/23.6, 24.1; 435/320.1, 419

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, CAPLUS
search terms: anf?, ampk?, plant, tobacoo, potato, tomato, rice, beet, bean, maize, glucose, kinase, sucrose, promoter?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	PURCELL et al. Antisense expression of a sucrose non-fermenting-1-related protein kinase sequence in potato results in decreased expression of sucrose synthase in tubers and loss of sucrose-inducibility of sucrose synthase transcripts in leaves. Plant J. 1998, Vol 14, No. 2, pages 195-202, see page 196, page 198, page 200.	20-21, 24-25, 28, 30 22-23, 26-29
Y	MURANAKA et al. Characterization of Tobacco Protein Kinase NPK5, a Homolog of Saccharomyces cerevisiae SNF1 That Constitutively Activates Expression of the Glucose-Repressible SUC2 Gene for a Secreted Invertase of S. cerevisiae. Mol. Cell. Biol. May 1994, Vol. 14, No. 5, pages 2958-2965, see page 2960.	20-22, 24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 01 NOVEMBER 2000	Date of mailing of the international search report 18 DEC 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ANNE R. KUBELIK Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/19981

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MONGER et al. Analyses of transgenic sugar beet hairy roots containing an antisense SNF1-related protein kinase sequence. J. Exp. Bot. 1995, Vol. 46, No. SUPPL, page 41.	18-24, 28-30
Y	MAN et al. Manipulation of SNF1-related protein kinase activity in transgenic potato. J. Exp. Bot. 1995, Vol. 46, No. SUPPL, page 35.	18, 20-21, 23-26
X Y	YANG et al. Expression of a SNF4-like protein kinase-related gene in tomato seeds. Plant Physiol. 1997, Vol. 114, No. 3, page 270.	1 2-17
X Y	Database Genbank on STN. (Accession No. U83797) LAKATOS et al. 'Nucleotide Sequence of a cDNA Clone Encoding an SNF1 Protein Kinase Homolog from Solanum tuberosum.' Plant Physiol. 1997, Vol. 117, page 1004.	18 19-24
Y	Database Genbank on STN. (Accession No. U40713) ABE et al. 'A cDNA Clone Encoding Yeast SNK4-Like Protein from Phaseolus vulgaris L.' Plant Physiol. 1996, Vol. 110, page 336.	2-17
Y	US 4,943,674 A (HOUCK et al) 24 July 1990, col 2, lines 20-29, and col 6, line 64 - col 7, line 6.	31

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/19981

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/19981

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

I. Claims 1-17, drawn to an isolated nucleic molecule encoding a plant SNF4 protein and methods for its use to transform plants for altered sugar metabolism.

II. Claims 18-30, drawn to an isolated nucleic acid molecule encoding a plant SNF1 protein and methods for its use to transform plants.

III. Claim 31, drawn to an isolated SNF4 promoter ligated to a heterologous structural gene.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Lakatos et al, 1997, teaches an isolated nucleic acid molecule encoding a polypeptide with 95% identity to SEQ ID NO:4. Thus, the claims do not constitute an advance over the prior art which would constitute a special technical feature. Furthermore, the claims are not drawn to a single nucleic acid or single amino acid sequence, but are instead drawn to a multitude of sequence variants. Thus, the claims are not limited to a single special technical feature.

The isolated SNF4 gene of Group I, a first product, requires a particular nucleic acid sequence encoding a particular amino acid sequence whose protein has a particular biological activity, each not required by any other group.

The isolated SNF1 gene of Group II, a second product, requires a particular nucleic acid sequence encoding a particular amino acid sequence whose protein has a particular biological activity, each not required by any other group.

The SNF structural genes and methods of plant transformation therewith of Groups I and II require methods for assaying altered sugar metabolism not required by Group III.

The isolated SNF4 promoter and heterologous structural gene of Group III, a third product, requires an SNF4 promoter and heterologous structural gene not involved in sugar metabolism, such as genes encoding antibiotic resistance or herbicide resistance, and methods for assaying the phenotypic effects of these structural genes, each not required by any other group.